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TITLE OF THE INVENTION (280 characters max)

A METHOD FOR IDENTIFYING ENZYME INHIBITORS

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A METHOD FOR IDENTIFYING ENZYME INHIBITORS

BACKGROUND OF THE INVENTION

Protein kinases represent one of the largest protein super-families in eukaryotes. Based on functional classification of human genes, the number of protein kinases encoded in the human genome is estimated to be in excess of 500 but less than 1000, which comprise about 3% of the total human genes (Venter, *et al., Science*, 291:1304-1351(2001)). Protein kinases play important roles in signal transduction pathways and in many cell regulatory processes such as cell division and differentiation, development, oncogenesis, cell survival and apoptosis. In addition, the genes encoding many protein kinases are located upstream or downstream of many of the epidemiologically relevant genes, underscoring them as targets for therapeutic intervention. It is therefore not surprising that protein kinases have emerged as attractive targets for identification and development of novel therapeutic agents for many disease indications.

Sequence alignment studies have revealed that most protein kinases share a common core of about 270 amino acids (Hank, S.K., and Hunter, T., *The Protein Kinase Facts Book*, Ed G. Kardie and S. Hanks, Academic Press, NY. (1995)). Studies comparing crystal structures of kinases have shown that the core structure of all kinases adopts a common fold (Hanks and Hunter, 1995). Most noteworthy, the regions in the N-terminal domain involved in ATP binding and the C-terminal domain involved in protein substrate recognition adopt similar conformation in most kinases as revealed by their crystal structures in their active forms. The ATP binding site is situated at the interface of the N-terminal and C-terminal lobes.

Structural studies shown that most of the small molecule kinase inhibitors studied so far bind to the Mg-ATP complex binding pocket (Garcia-Echeverria *et al., Med. Res. Rev.*, 20:28-57 (2000)). As a result, considerable progress has been made towards the synthesis of potent and selective ATP site directed protein kinase inhibitors by modulating and fine-tuning the chemical templates (Garcia-Echeverria *et al.*, 2000). Together these studies have refuted the accepted idea that the ATP binding cleft is not an ideal target for development of potent, specific inhibitors.

p-Flurosulfonylbenzoyl 5'-adenosine (FSBA) is an ATP-affinity reagent that is effective in covalent modification of nucleotide-binding sites in a variety of protein

kinases (Kamps, *et al.*, *Nature* 310:589-592 (1984); Scoggins, *et al.*, *Biochemistry* 35:9197-9203 (1996); Young, *et al.*, *J. Biol. Chem.* 272: 12116-12121 (1997); Fox, *et al.*, *FEBS Letts.* 461:323-328 (1999)). Structurally FSBA is similar to ATP except for the presence of flurosulfonylbenzoyl moiety in place of the three phosphates of ATP. FSBA has been used to selectively label and characterize a number of protein kinases. Peptide mass finger printing studies carried out on a panel of protein kinases have shown that FSBA binds irreversibly to the side chain of a critical, conserved lysine found in the ATP binding site (Kamps, *et al.*, 1984; Zoller *et al.*, *J. Biol. Chem.* 256:10837-10842 (1981)). ^{14}C-labeled FSBA (Fox, *et al.*, 1999; Buhrow, *et al.*, . *Biol. Chem.* 258:7824-7827 (1983)) and FSBA-specific antibodies (Parker, *FEBS Letts.* 334:347-350 (1993); T'Jampens, *et al.*, *FEBS Letts.* 516:20-26 (2002)) have been used to identify and characterize protein kinases from cell lysates, although these reagents because of their limitations have not been effectively used for inhibitor screening (T'Jampens, *et al.*, 2002).

Liquid chromatography/mass spectrometry (LC/MS) technique has been valuable in monitoring covalent modification of natural and recombinantly expressed proteins (Feng, *et al.*, *Anal. Chem.* 73:5691-5697 (2001)). These techniques have also been routinely used in drug metabolism and pharmacokinetics studies (Feng, *et al.*, 2001), but their use as screening tools has not been widely explored.

Accordingly, a method for identifying compounds that inhibit kinases is greatly needed. In addition, a method for rapidly profiling protein kinases is also greatly needed.

SUMMARY OF THE INVENTION

One embodiment of the present invention is to provide a method for identifying a compound that inhibits an enzyme having an ATP binding site comprising contacting a composition of the enzyme, an analyte capable of binding to the ATP site of the enzyme, and a test compound, and detecting whether the test compound inhibits the analyte from binding the ATP binding site of the enzyme.

A further embodiment of the present invention, provides a method for identifying a compound that inhibits a kinase having an ATP binding site comprising the steps of contacting a composition comprising the kinase and an analyte that binds to an ATP binding site of the kinase; detecting binding of the analyte to the ATP binding site; contacting a composition comprising the kinase, the analyte, and a test compound, and

detecting whether the test compound inhibits the analyte from binding the ATP binding site.

In another embodiment of the present invention, a method is provided for identifying a test compound that inhibits a kinase with an ATP-binding site comprising the steps of contacting a composition comprising the kinase and test compound; contacting the composition comprising the kinase and the test compound with an analyte; and detecting whether the test compound inhibits the analyte from binding the kinasé's ATP binding site.

Also provided in the instant invention is a method wherein detecting the binding of the test compound to the kinase comprises using mass spectrometry.

A still further embodiment of the invention provides a method wherein the test compound is a competitive inhibitor of the analyte.

Another embodiment of the invention is a method wherein the analyte is FSBA.

DETAILED DESCRIPTION OF THE INVENTION

Because FSBA binds covalently in the ATP binding pocket of protein kinases, LC/MS has utility in screening ATP competitive protein kinase inhibitors. This invention demonstrates that using purified recombinant kinases and FSBA as an activity-based probe (ABP), LC/MS provides a general, rapid and reproducible means to screen ATP competitor inhibitors of protein kinases.

In the present invention, autoradiography and LC/MS techniques are used to evaluate FSBA as an activity-based probe for protein kinases. The work presented here demonstrates FSBA's utility as an activity-based probe and LC/MS's usefulness as a screening tool for the selection of ATP competitor protein kinase inhibitors. Compared to fluorography, which takes days to weeks for evaluation, LC/MS allows rapid -10 min – detection of the inhibitor screening. Furthermore, with the advent of a new integrated, 10-pump, eight channel, parallel LC/MS (Feng *et al.*, 2001), this method has potential to be used in a high throughput mode as well.

Thus, one embodiment of the present invention is to provide a method for identifying a compound that inhibits an enzyme having an ATP binding site comprising contacting a composition of the enzyme, an analyte capable of binding to the ATP site of the enzyme, and a test compound, and detecting whether the test compound inhibits the analyte from binding the ATP binding site. In one aspect of the invention, the enzyme is

a kinase. In another aspect of the invention, the test compound is a competitive inhibitor of the analyte. In yet another aspect of the invention, the analyte is p-flurosulfonylbenzoyl 5'-adenosine (FSBA). In yet another aspect of the invention, the enzyme comprises a conserved lysine in the ATP binding site. In yet another aspect of the invention, the analyte is bound to the conserved lysine. Detecting whether the test compound inhibits the analyte from binding to the enzyme can be done by mass spectrometry, a protease assay, or a kinase assay.

In another embodiment of the present invention, a method is provided for identifying a compound that inhibits a kinase having an ATP binding site comprising the steps of contacting a composition comprising the kinase and an analyte that binds to an ATP binding site of the kinase; detecting binding of the analyte to the ATP binding site; contacting a composition comprising the kinase, the analyte, and a test compound, and detecting whether the test compound inhibits the analyte from binding the ATP binding site. In another aspect of the invention, the enzyme has a conserved lysine in the ATP binding site. In yet another aspect of the invention, the analyte is bound to the conserved lysine. In another aspect of the invention, the binding of the analyte to the ATP binding site can be performed by mass spectrometry, a protease assay or a kinase assay. In another aspect of the invention, the test compound is a competitive inhibitor of the analyte. The analyte may be p-flurosulfonylbenzoyl 5'-adenosine (FSBA).

In another embodiment of the present invention, a method is provided for identifying a test compound that inhibits a kinase having an ATP binding site comprising the steps of contacting a composition comprising the kinase and test compound; contacting the composition comprising the kinase and the test compound with an analyte; and detecting whether the test compound inhibits the analyte from binding the kinase's ATP binding site. In another aspect of the invention, the enzyme has a conserved lysine in the ATP binding site. In another aspect of the invention, the analyte is bound to the conserved lysine. Detection of whether the test compound inhibits the analyte from binding the kinase's ATP binding site can be done by using mass spectrometry, a protease assay, or a kinase assay. In yet another aspect of the invention, the test compound is a competitive inhibitor of the analyte. The analyte may be p-flurosulfonylbenzoyl 5'-adenosine (FSBA).

The following examples illustrate various aspects of this invention. These examples do not limit the scope of this invention which is defined by the appended claims.

EXAMPLES

Purified protein kinases were used in the following examples. Where indicated proteins and chemicals were obtained from Sigma. ^{14}C-labeled FSBA was obtained from Perkin Elmer Life Sciences. Autoradiography on ^{14}C-FSBA labeled protein kinases was carried out as described (Buhrow *et al.*, 1983; Fox *et al.*, 1997). The dried gels were exposed to film at –80 C for 2-4 weeks. MS analysis of purified proteins and FSBA-modified protein kinases were carried out as described (Feng *et al.*, 2001).

For FSBA labeling, purified proteins (0.2-0.5 mg/ml) were incubated with 10 μM FSBA (in 2.5% DMSO) at room temperature. For time dependent modification studies, 10 μl aliquot was removed at an indicated time and was mixed with 50 μl of 0.1% TFA and injected on LC/MS as described before (Feng *et al.*, 2001). For ATP protection experiments, purified kinases were coincubated with 10 μM FSBA and various amounts of ATP and $MgCl_2$ for 1-2 h at room temperature. For staurosporine protection experiments, purified protein kinases were coincubated with 10 μM FSBA with varied amounts of staurosporine (0.1μM – 10μM) for 1-2 h at room temperature. Samples were subjected to LC/MS as described above.

Autoradiography and LC/MS techniques were used to evaluate the utility of FSBA as an activity-based probe for protein kinases. The work presented here demonstrates FSBA's utility as an activity-based probe and that LC/MS can be used as a screening tool for the selection of ATP competitor protein kinase inhibitors.

Example 1: Affinity labeling of FSBA detected by autoradiography

FSBA, an ATP analogue, is an affinity label that covalently labels most protein kinases by binding to the ATP pocket. For initial FSBA labeling studies, recombinantly expressed and purified kinase domain of the transforming growth factor (TGF)-β type I receptor (activin receptor-like kinase, ALK5; Laping *et al., Mol. Pharmacol.* 62:58-64 (2002)) was used. TGF-β acts through ALK5 to activate various mediators. Because TGF-β is a potent stimulus for extracellular matrix synthesis, inhibition of ALK5 activity may be beneficial in fibrotic disorders (Kanzler, *et al., Am. J. Physiol.* 39:G1059-G1068 (1999); Laping, *et al.* 2002).

^{14}C-labeled FSBA was incubated with purified preparations of ALK5 (Laping *et al.*, 2002) for one hour at room temperature. The samples were either heated at 95°C or kept on ice for 10 min prior to the addition of ^{14}C -FSBA. Samples were subjected to SDS-PAGE and were either processed for autoradiography or stained with Coomassie blue. Autoradiography results indicated that FSBA labeled unheated ALK5 and CDK2 but did not label heat-denatured ALK5. In cold competition experiments, cold FSBA competed with the binding of ^{14}C-labeled FSBA to ALK5 kinase. According to the Coomassie blue staining pattern of the unheated and heated samples, FSBA binds only to the native-like conformations of ALK5. Similar results were obtained for a panel of other kinases.

Earlier studies have shown that ATP competes with FSBA modification of many kinases (Kamps *et al.*, 1984; Fox *et al.*, 1999; T'Jampens *et al.*, 2002). To extend these studies to ALK5, purified ALK5 was incubated for 1 hour with ~20 μM ^{14}C FSBA and 1 mM or 10 mM ATP. The autoradiography and Coomassie blue staining of the gel indicate that most of the labeling was blocked by 1 mM ATP and 10 mM ATP offered complete protection. These results indicate that FSBA and ATP compete for the same binding pocket in ALK5.

Example 2: LC/MS as an alternative technique to follow affinity labeling by FSBA

Together the heat denaturation and ATP protection results indicated that FSBA has potential to be an activity-based probe for kinase profiling studies. Recently flurophosphonate/flurophosphate (FP) derivatives were used in profiling serine hydrolases (Liu *et al.*, *Proc. Natl. Acad. Sci. USA* 96:14694-14699(1999); Kidd *et al.*, *Biochemistry* 40, 4005-4015 (2001)). The FP inhibitors display reactivity against a majority of serine hydrolases in an activity-dependent manner. Since FSBA reacts with a panel of kinases by binding to active sites, and its labeling appears to be dependent on the conformational/activity state of the kinase FSBA was used as an ABP for selecting ATP competitive inhibitors of kinases.

Although the radioactive form of FSBA, which is commercially available, can be used for kinase profiling studies, its detection by fluorography requires several days to weeks. In addition handling and disposal of radioactive material also makes it a less attractive reagent for screening studies. Since FSBA modification of kinases is covalent,

which is expected to be stable to the LC/MS technique, the LC/MS technique was used as part of this invention as an alternative to autoradiography.

Time-dependent reactions were performed to assess FSBA modification of kinases by LC/MS. Purified ALK5 was incubated with FSBA at room temperature and aliquots of samples taken out at defined time intervals were subjected to LC/MS. Deconvulated mass spectrum profile revealed the time-dependent covalent modification of ALK5 with FSBA. Purified ALK5 gave a single predominant peak with a molecular mass of 34,974 Da, expected for the baculovirus-expressed unphosphorylated form of ALK5 protein (Laping *et al.*, 2002). Incubation in the presence of FSBA caused a shift in molecular mass to 35,405 Da in a time-dependent manner. By 60 minutes, the parent peak was completely converted to a new peak with increased mass of 433 Da. Likewise, the covalent modification of ALK4 and CDK2, two other recombinantly expressed and purified kinases was also nearly completed in about 1 hour. This modification was demonstrated by an increase (433 Da) in the molecular mass of FSBA-treated ALK4 and CDK2 proteins.

Under the same conditions, FSBA did not modify trypsin and BSA (kinases that do not contain an ATP binding site), indicating FSBA's selective reactivity to ATP-requiring kinases tested under these studies. The difference in the unmodified and FSBA-modified forms of each of the kinases tested accounts for the presence of covalently attached sulfonyl benzoyl adenosine moiety with the removal of fluorine atom. These results are analogous to the affinity labeling of P38γ by FSBA as reported by Fox *et al.* (1999). Based on their LC/MS studies, both the unphosphorylated and the phosphorylated forms of P38γ were modified by FSBA as seen by increase in mass of 433 Da of the FSBA-treated protein.

Example 3: ATP competes with FSBA modification

Autoradiography studies showed that ATP competes with FSBA modification of ALK5 kinases. To follow ATP protection effect by LC/MS and to extend the ALK5 results to other kinases, purified ALK5, ALK4 and CDK2 kinases were coincubated the presence of FSBA and varying amounts (0.1μM – 1mM) of ATP as presented in Table 1.

Table 1

Protein Kinase	Additives	Protein Species (Da) (relative percentage)
ALK5	none	34,972 Da (100%)
ALK5	FSBA (10 µM)	35,405 Da (100%)
ALK5	FSBA (10 µM) plus ATP (50 µM)	35,405 Da (100%)
ALK5	FSBA (10 µM) plus ATP (100 µM)	35,405 Da (70%), 34,972 Da (30%)
ALK5	FSBA (10 µM) plus ATP (500 µM)	34,972 Da (100%)
ALK4	none	35,025 Da (100%)
ALK4	FSBA (10 µM)	35,459 Da (100%)
ALK4	FSBA (10 µM) plus ATP (50 µM)	35,459 Da (95%), 35,025 Da (5%)
ALK4	FSBA (10 µM) plus ATP (100 µM)	35,459 Da (70%), 35,025 Da (30%)
ALK4	FSBA (10 µM) plus ATP (500 µM)	35,025 Da (100%)
CDK2	none	33,971 Da (100%)
CDK2	FSBA (10 µM)	34,404 Da (100%)
CDK2	FSBA (10 µM) plus ATP (1 mM)	34,404 Da (80%), 33,971 Da (20%)
CDK2	FSBA (10 µM) plus ATP (5 mM)	34,404 Da (10%), 33,971 Da (90%)
CDK2	FSBA (10 µM) plus ATP (10 mM)	33,971 Da (100%)

ATP protected covalent modification of both ALK5 and ALK4 by FSBA in a concentration-dependent manner; most of the covalent modification was inhibited in the presence of 0.5 mM ATP. Likewise ATP protected labeling of CDK2 with FSBA in a concentration-dependent manner, although 5 mM ATP was necessary to obtain complete protection. Similar results were obtained by autoradiography using ^{14}C –labeled FSBA. The ATP protection experiment suggests that the affinity of ATP for ALK5 and ALK4 is significantly higher than that for CDK2. These results suggest that LC/MS in combination with FSBA modification provides a rapid way to probe the ATP binding pocket of recombinantly expressed kinases.

Example 4: Staurosporine competes with FSBA labeling

ATP competitor small molecule protein kinase inhibitors, which also encompass the ATP binding pocket, were determined to prevent covalent modification of kinases by FSBA. Staurosporine, a microbial alkaloid, is a potent, but non-specific protein kinase

inhibitor (Rueegg *et al.*, *Trends Pharmacol. Sci.* 10:218 (1989); Garcia-Echeverria *et al.*, 2000). It inhibits a number of kinases (Jacobson ,*et al.*, *J. Cell Biol.* 133:1041-1051 (1996); Schnier, *et al.*, *Proc. Natl. Acad. Sci. USA* 1041-1051 (1996)), including CDK2 (DeBondt, *et al.*, *Nature* 363:595-602 (1993); Lawrie ,*et al.*, *Nature Structural Biology* 4:796-801 (1997); Zhao, *et al.*, *J. Biol. Chem.* 277:46609-46615 (2002)) and ALK5 (N. Laping, unpublished results), with IC_{50} values in the nanomolar range. Crystal structures of CDK2 and Chk1 kinases complexed with staurosporine have been reported (Lawrie, *et al.*, 1997; Zhao, *et al.*, 2002). In both structures, staurosporine was shown to bind in the ATP binding cleft with the terahydropyran ring in a boat conformation.

To evaluate protective effects of staurosporine, purified ALK5, ALK4, CDK2, and kinases were coincubated with FSBA and increasing amounts of staurosporine and samples were analyzed by LC/MS. LC/MS profiles of each of the kinases tested in the presence of FSBA and staurosporine demonstrated that staurosporine abrogated FSBA labeling in concentration-dependent manner and the modification was virtually blocked by the addition of 10 uM staurosporine.

Table 2

Protein Kinase	Additives	Protein Species (Da) (relative percentage)
ALK5	none	34,972 Da (100%)
ALK5	FSBA (10 µM)	35,405 Da (100%)
ALK5	FSBA (10 µM) plus staurosporine (0.1 µM)	35,405 Da (90%), 34,972 Da (10%)
ALK5	FSBA (10 µM) plus staurosporine (1 µM)	35,405 Da (70%), 34,972 Da (30%)
ALK5	FSBA (10 µM) plus staurosporine (10 µM)	34,972 Da (100%)
ALK4	none	35,025 Da (100%)
ALK4	FSBA (10 µM)	35,459 Da (100%)
ALK4	FSBA (10 µM) plus staurosporine (0.1 µM)	35,459 Da (100%)
ALK4	FSBA (10 µM) plus staurosporine (1 µM)	35,459 Da (80%), 35,025 Da (20%)
ALK4	FSBA (10 µM) plus staurosporine (10 µM)	35,025 Da (100%
CDK2	none	33,971 Da (100%)
CDK2	FSBA (10 µM)	33,404 Da (100%)
CDK2	FSBA (10 µM) plus staurosporine (0.1 µM)	33,404 Da (100%)
CDK2	FSBA (10 µM) plus staurosporine (1 µM)	33,404 Da (70%), 33,971 Da (30%)
CDK2	FSBA (10 µM) plus staurosporine (10 µM)	33,971 Da (100%)

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We Claim:

1. A method for identifying a compound that inhibits an enzyme having an ATP binding site comprising the steps of:

(a) contacting a composition comprising the enzyme having an ATP binding site, an analyte capable of binding to the ATP site of said enzyme, and a test compound, and

(b) detecting whether said test compound inhibits said analyte from binding said ATP binding site.

2. The method of claim 1, wherein said enzyme is a kinase.

3. The method of claim 1 wherein said test compound is a competitive inhibitor of said analyte.

4. The method of claim 1 wherein said analyte is p-flurosulfonylbenzoyl 5'-adenosine (FSBA).

5. The method of claim 1, wherein the enzyme comprises a conserved lysine in the ATP binding site.

6. The method of claim 5, further comprising binding the analyte to the conserved lysine.

7. The method of claim 1 wherein said detecting step comprises using mass spectrometry.

8. The method of claim 1, wherein said detecting step comprises using a protease assay.

9. The method of claim 1, wherein the said detecting step comprises using a kinase assay.

10. A method for identifying a compound that inhibits a kinase having an ATP binding site comprising the steps of:

(a) contacting a composition comprising a kinase and an analyte that binds to an ATP binding site of said kinase,

(b) detecting binding of said analyte to said ATP binding site,

(c) contacting a composition comprising said kinase, said analyte, and a test compound, and

(d) detecting whether said test compound inhibits said analyte in step (c) from binding said ATP binding site.

11. The method of claim 10, wherein the kinase comprises a conserved lysine in the ATP binding site.

12. The method of claim 11, further comprising binding the analyte to the conserved lysine.

13. The method of claim 10, wherein said detecting step comprises using mass spectrometry.

14. The method of claim 10, wherein said detecting step comprises using a protease assay.

15. The method of claim 10, wherein the said detecting step comprises using a kinase assay.

16. The method of claim 10, wherein said test compound is a competitive inhibitor of said analyte.

17. The method of claim 10, wherein said analyte is p-flurosulfonylbenzoyl 5'-adenosine (FSBA).

18. A method for identifying a test compound that inhibits a kinase having and ATP binding site comprising the steps of:

(a) contacting a composition comprising the kinase and test compound,

(b) contacting a composition comprising said kinase and said test compound with an analyte and

(c) detecting whether said test compound inhibits said analyte in step (b) from binding said ATP binding site.

19. The method of claim 18, wherein the kinase comprises a conserved lysine in the ATP binding site.

20. The method of claim 19, further comprising binding the analyte to the conserved lysine.

21. The method of claim 18, wherein said detecting step comprises using mass spectrometry.

19. The method of claim 18, wherein said detecting step comprises using a protease assay.

20. The method of claim 18, wherein the said detecting step comprises using a kinase assay.

22. The method of claim 18, wherein said test compound is a competitive inhibitor of said analyte.

23. The method of claim 18, wherein said analyte is p-flurosulfonylbenzoyl 5'-adenosine (FSBA).

PU60144P

Abstract

The present invention provides a method for identifying compounds that inhibit kinases. In addition, a method for rapidly profiling protein kinases is also provided.